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Milk *Streptococcus uberis* PCR Detection Kit — Product Insert

Product # 30800

Mastitis is the single most costly disease of dairy cattle resulting in the reduction of milk yield and quality. The inflammation of the utter is mainly caused by infection of various bacteria. *Streptococcus uberis* is a gram-positive bacterium that is known worldwide as an environmental pathogen responsible for a high proportion of cases of mastitis in lactating cows and is also the predominant organism isolated from mammary glands during the non-lactating period. Often it is resistant to treatment and causes persistent high somatic cell counts without clinical mastitis.

Principle of the Test and Product Description

Norgen's Milk *Streptococcus uberis* PCR Detection Kit constituents a ready-to-use system for the isolation without enrichment and the detection of *S. uberis* using end-point PCR. The kit first allows for the isolation of bacterial DNA from milk samples using spin-column chromatography based on Norgen's proprietary resin. The DNA is isolated free from inhibitors, and can then be used as the template in a PCR reaction for *S. uberis* detection using the provided *S. uberis* Master Mix. The *S. uberis* Master Mix contains reagents and enzymes for the specific amplification of a 324 bp region of the *S. uberis* genome. In addition, Norgen's Milk *Streptococcus uberis* PCR Detection Kit contains a second heterologous amplification system to identify possible PCR inhibition and/or inadequate isolation. The amplification and the detection of *Isolation Control (IsoC)* or the *PCR control (PCRC)* does not reduce the detection limit of the analytical *S. uberis* PCR. This kit is designed to allow for the testing of 24 samples.

Kit Components:

Component	Contents
Digestion Buffer	3 mL
Lysis Solution	12 mL
Binding Solution	4 mL
Wash Solution I	15 mL
Wash Solution II	5 mL
Elution Buffer	8 mL
Proteinase K	6 mg
Lysozyme	60 mg
Mini Filter Spin Columns	25
Collection Tubes	25
Elution tubes (1.7 mL)	25
S. uberis 2x PCR Master Mix	0.35 mL
S. uberis Isolation Control (IsoC)**[a]	0.4 mL
S. uberis Positive Control (PosC)**[b]	0.1 mL
Nuclease Free-Water	1.25 mL
Norgen's DNA Marker	0.1 mL
Product Insert	1

** IsoC = Isolation Control ; PosC= Positive Control*
[a] The positive control is purified S. uberis genomic DNA fragments.
[b] The isolation control is a cloned PCR product.

Customer-Supplied Reagents and Equipment

- Disposable powder-free gloves
- Benchtop microcentrifuge
- Micropipettors
- Sterile pipette tips with filters
- PCR tubes
- 96 – 100% ethanol
- 37°C incubator
- 55°C incubator

Storage Conditions and Product Stability

All buffers should be kept tightly sealed and stored at room temperature (15-25°C). Buffers can be stored for up to 1 year without showing any reduction in performance.

The Lysozyme should be stored at -20°C upon arrival, and the Digestion Buffer should be stored at -20°C after addition of the Lysozyme. The Proteinase K should be stored at -20°C upon arrival and after reconstitution. These reagents should remain stable for at least 1 year when stored at these conditions.

The *S. uberis* 2x PCR Master Mix, *S. uberis* Isolation Control (IsoC), and *S. uberis* Positive Control (PosC) should be kept tightly sealed and stored at -20°C. These can be stored for up to 1 year without showing any reduction in performance. Repeated thawing and freezing (> 2 x) of these reagents should be avoided, as this may reduce the sensitivity. If the reagents are to be used only intermittently, they should be frozen in aliquots.

General Precautions

The user should exercise the following precautions when using the kit:

- Use sterile pipette tips with filters.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Work quickly on ice.

Quality Control

In accordance with Norgen's ISO 9001 and ISO 13485-certified Quality Management System, each lot of Norgen's Milk *Streptococcus uberis* PCR Detection Kit, including the *S. uberis* 2x PCR Master Mix, *S. uberis* Isolation Control (IsoC) and *S. uberis* Positive Control (PosC) are tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

Norgen's Milk *S. uberis* PCR Detection Kit is designed for research purposes only. It is not intended for human or diagnostic use.

Product Warranty and Satisfaction Guarantee

NORGEN BIOTEK CORPORATION guarantees the performance of all products in the manner described in our product manual. The customer must determine the suitability of the product for its particular use.

Safety Information

Biosafety level 2 practices are recommended for works involving *Streptococcus uberis*. Ensure the appropriate containment equipment and facilities are used for activities involving cultures or potentially infectious clinical materials. Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when working with chemicals. For more information, please consult the appropriate Material Safety Data Sheets (MSDSs). These are available as convenient PDF files online at www.norgenbiotek.com.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

The **Binding Solution** and **Wash Solution I** contain guanidine salts, and should be handled with care. Guanidine salts form highly reactive compounds when combined with bleach, thus care must be taken to properly dispose of any of these solutions.

If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

Protocol

A. *Streptococcus uberis* Genomic DNA Isolation

Precaution: All samples must be treated as potentially infectious material.

Important Notes Prior to Beginning Protocol:

- A variable speed centrifuge should be used for maximum kit performance. If a variable speed centrifuge is not available a fixed speed centrifuge can be used, however reduced yields may be observed.
- Preheat an incubator or heating block to 37°C and another to 55°C.
- Reconstitute the **Proteinase K** in 300 µL of molecular biology grade water, aliquot into small fractions and store the unused portions at -20°C until needed.
- Add the provided amount of **Digestion Buffer** to the tube containing the **Lysozyme**, and mix well. Aliquot the **Digestion Buffer** into small fractions and store the unused portions at -20°C until needed.
- Prepare a working concentration of **Wash Solution II** by adding 15 mL of 96 - 100% ethanol (to be provided by the user) to the supplied bottle containing concentrated **Wash Solution II**. This will give a final volume of 20 mL. The label on the bottle has a box that can be checked to indicate that ethanol has been added.
- ***S. uberis* Isolation Control (*IsoC*)**
  - A *S. uberis* Isolation Control (*IsoC*) is supplied. This allows the user to control the DNA isolation procedure. For this assay, add the *S. uberis* Isolation Control (IsoC) to the lysate during the isolation procedure
  - The *S. uberis* Isolation Control (*IsoC)* must not be added to the sample material directly.
  - Do not freeze and thaw the *S. uberis* Isolation Control (IsoC) more than 2 times.
  - The *S. uberis* Isolation Control (IsoC) must be kept on ice at all times during the isolation procedure.
- **Milk Samples**
  - Freshly collected milk samples or enriched samples are recommended
  - Frozen milk samples may be used, but note that the sensitivity of detection may be decreased
  - Milk samples collected into preservatives (such as bronopol) are not recommended
- The PCR components of the *Streptococcus uberis* PCR Detection Kit should remain at -20°C until DNA is extracted and ready for PCR amplification.

1. Lysate Preparation

a. Vortex the milk sample for 10 to 15 seconds or invert several times to mix.
b. Aliquot a maximum of 1 mL of milk into a microcentrifuge tube.

Note: Up to 1 mL of milk is recommended for normal milk samples. **For samples with high leukocyte counts, up to 200 µL of milk sample is recommended**. If the sample is

very viscous and difficult to pipette, pass the sample through an 18-gauge syringe a few times to reduce the viscosity.

c. Centrifuge at 14,000 x g (~14,000 RPM) for 3 minutes.
d. Pour off the supernatant by quickly inverting the tube and gently tapping it against the wall of the waste container. This tapping is to ensure that the creamy layer present on the top of the milk sample after centrifugation is removed. Clean any remaining white solid from the microcentrifuge tube wall by using a clean cotton swab. Ensure that the pellet is not dislodged.
e. Resuspend the pellet in 100 µL of **Digestion Buffer**. Incubate at 37°C for 45 minutes.

Note: Ensure that the provided Lysozyme has been added to the Digestion Buffer.

f. After incubation, add 300 µL of **Lysis Solution** and 10 µL of reconstituted **Proteinase K** to the digestion mixture and mix well by vortexing.
g. Incubate the lysate at 55°C for 45 minutes. Mix the lysate occasionally by vortexing.

2. Sample Binding to Column

a. After incubation, add 40 µL of **Binding Solution,** 10 µL of ***S. uberis* Isolation Control (*IsoC)*** and 180 µL of 96-100% ethanol to the lysis mixture, and mix by vortexing.

Note: Ensure that the ***S. uberis* Isolation Contro (*IsoC)*** is added for subsequent control detection in the PCR protocol

b. Spin the sample for 10 seconds at 14,000 x g (~14,000 RPM). A thin layer of lipid may form on the top of the aqueous phase. Using a pipette, carefully transfer the clear aqueous phase only to a spin column that has been attached to a collection tube.
c. Centrifuge the column assembly for 3 minutes at 14,000 x g (~14,000 RPM) to bind the bacterial DNA.

Note: If all the liquid does not pass through the column, spin for an additional 2 minute at 14,000 x g (~14,000 RPM). If a small amount of liquid still remains on the top the column, proceed to Step **3a** with the addition of **Wash Solution I**.

3. Column Wash

a. Apply 500 µL of **Wash Solution I** to the column and centrifuge for 2 minutes at 14,000 x g (~14,000 RPM).
b. Discard the flowthrough and reassemble the column and the collection tube.
c. Apply 500 µL of **Wash Solution II** to the column and centrifuge again for 2 minutes at 14,000 x g (~14,000 RPM).

Note: Ensure the appropriate amount of ethanol has been added to **Wash Solution II**.

d. Discard the flowthrough and reassemble the column and the collection tube. Centrifuge for 2 minutes at 14,000 x g (~14,000 RPM) to ensure the resin is completely dry.
e. Discard the collection tube.

4. DNA Elution

a. Transfer the spin column to a provided 1.7 mL Elution tube.
b. Apply 75 µL of **Elution Buffer** to the column and centrifuge at 2,600 x g (~6,000 RPM) for 2 minutes.
c. Spin for an additional 2 minutes at 14,000 x g (~14,000 RPM) to complete the DNA elution.

B. *Streptococcus uberis* PCR Assay Preparation

Notes:
- Before use, suitable amounts of all PCR components should be completely thawed at room temperature, vortexed and centrifuged briefly.
- The amount of *S. uberis* 2X PCR Master Mix provided is enough for up to 32 PCR reactions (24 sample PCR, 4 positive control PCR and 4 no template control PCR).
- For every PCR run, one reaction containing ***S. uberis* Positive Control** (***PosC***) and one reaction as no template control must be included for proper interpretation of results.
- The recommended minimum number of DNA samples tested per PCR run is 6.

1. Prepare the PCR for sample detection as shown in Table 1 below. The recommended amount of sample DNA to be used is 5 µL. However, A volume between 1 and 10 µL of sample DNA may be used as template. Adjust the final volume of the PCR reaction to 20 µL using the Nuclease-Free Water provided.

Table 1. PCR Assay Preparation

PCR Components	Volume Per PCR Reaction
***S. uberis* 2X PCR Master Mix**	**10 µL**
Sample DNA	**1 to 10 µL**
Nuclease-Free Water	**Up to 10 µL**
Total Volume	**20 µL**

2. For every PCR run, prepare **one** positive control PCR as shown in Table 2 below:

Table 2. PCR Positive Control Preparation

PCR Components	Volume Per PCR Reaction
***S. uberis* 2X PCR Master Mix**	**10 µL**
S. uberis Positive Control (PosC)	**10 µL**
Total Volume	**20 µL**

3. For every PCR run, prepare **one** no template control PCR as shown in Table 3 below:

Table 3. PCR Negative Control Preparation

PCR Components	Volume Per PCR Reaction
***S. uberis* 2X PCR Master Mix**	**10 µL**
Nuclease-Free Water	**10 µL**
Total Volume	**20 µL**

C. *Streptococcus uberis* PCR Assay Programming

1. Program the thermocylcer according to the program shown in Table 4 below.
2. Run PCR.

Table 4. *S. uberis* Assay Program

PCR Cycle	Step	Temperature	Duration
Cycle 1	**Step 1**	**95°C**	**3 min**
Cycle 2 (40x)	**Step 1**	**94°C**	**15 sec**
	Step 2	**60°C**	**30 sec**
	Step 3	**72°C**	**45 sec**
Cycle 3	**Step 1**	**72°C**	**5 min**
Cycle 4	**Step 1**	**4°C**	**∞**

D. *Streptococcus uberis* PCR Assay Results Interpretation

1. For the analysis of the PCR data, the entire 20 µL PCR Reaction should be loaded on a 1X TAE 1.7% Agarose DNA gel along with 10 µL of Norgen's DNA Marker (provided).
2. The PCR products should be resolved on the 1X TAE 1.7% Agarose gel at 150V for 30 minutes.
3. Sample results are provided below:

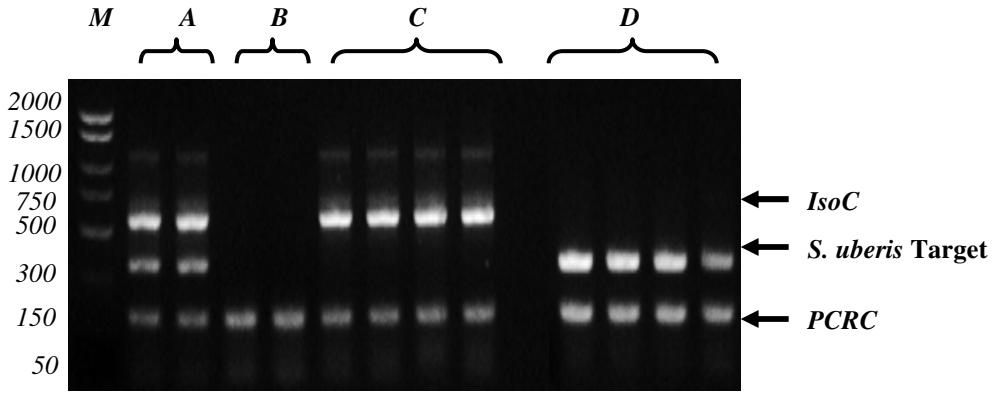

Figure 1: A representative 1X TAE 1.7% Agarose gel showing the amplification of *Streptococcus uberis* at different concentration (*S. uberis* Target). The size of the *S. uberis* target amplicon corresponds to 324 bp as represented by the provided DNA Marker (M). The size of the Isolation Control (*IsoC*) corresponds to 550bp as represented by the provided DNA Marker (M). The *S. uberis* 2X PCR Master Mix contains an a PCR Control (*PCRC*). The PCRC Controls for PCR inhibition. The size of the PCRC corresponds to 171bp as represented by the provided DNA Marker (*M*). The amplification from each lane is interpreted as follows:

Lane A: **Positive Control** or *S. uberis* **Detected** – All three PCR amplicons were detected
Lane B: No Template Control – Only ***S. uberis PCRC*** was detected
Lane C: *S. uberis* **Not Detected –** Detection ***Isolation Control (IsoC)*** and ***PCRC***, suggesting that the DNA isolation was successful but no ***S. uberis*** DNA was present in the sample
Lane D: *S. uberis* **Detected** – Lane D showed detection of both the ***S. uberis* Target** and ***PCRC*** but without ***IsoC.*** The results are still considered positive detection of *S. uberis.*

Table 5. Interpretation of PCR Assay Results

Input Type	IsoC Band (550 bp)	*S. uberis* Target Band (324 bp)	PCRC Band (171 bp)	Interpretation
Positive Control	X	X	X	Valid
Negative Control			X	Valid
Sample	X	X	X	Positive
Sample	X		X	Negative
Sample		X	X	Positive
Sample	X	X		Positive
Sample		X		Positive

** For results obtained that are not covered in Table 5 above, please refer to the Troubleshooting Section.

E. *Streptococcus uberis* PCR Assay Specificity and Sensitivity

- The specificity of Norgen's Milk *Streptococcus uberis* PCR Detection Kit is first and foremost ensured by the selection of the *S. uberis* -specific primers, as well as the selection of stringent reaction conditions. The primers were checked for possible homologies to all in GenBank published sequences by sequence comparison analysis. The specific detectability of all relevant strains has thus been ensured by a database alignment and by PCR amplification with the following bacteria commonly found in mastitis milk samples or contaminated milk samples:
    - *E coli*
    - *Streptococcus agalactiae*
    - *Sterptococcus dysgalactiae*
    - *Staphylococcus aureus*
    - *Listeria monocytogenes*
    - *Salmonella enterica*

F. Linear Range

- The linear range (analytical measurement) of Norgen's Milk *Streptococcus uberis* PCR Detection Kit was determined by analysing a dilution series of a *S. uberis* quantification standard ranging from 1 x 10^7 cfu/µl to 1 x 10^{-1} cfu/µl.
- Each dilution has been tested in replicates (n = 4) using Norgen's Milk *Streptococcus uberis* PCR Detection Kit on 1X TAE 1.7% Agarose gel.
- The linear range of Norgen's Milk *Streptococcus uberis* PCR Detection Kit has been determined to cover concentrations from 1 x 10^2 cfu/µl to at least 1 x 10^6 cfu/µl
- Under the conditions of the Norgen's Milk *Streptococcus uberis* DNA Isolation procedure, Norgen's Milk *Streptococcus uberis* PCR detection Kit covers a linear range from 1,000 cfu/mL milk to at least 1 x 10^7 cfu/mL milk.

Frequently Asked Questions

1. How many samples should be included per PCR run?

- Norgen's Milk *Streptococcus uberis* PCR Detection Kit is designed to test 24 samples. For every 6 samples, a non-template control and a Positive Control must be included. It is preferable to pool and test 6 samples at a time. If not, the provided Positive Control is enough to run 3 samples at a time.

2. How can I interpret my results if neither the PCR control (PCRC) nor the Isolation Control (IsoC) amplifies?

- If neither the PCR control nor the Isolation Control amplifies, the sample must be re-tested. If the positive control showed amplification, then the problem occurred during the isolation, where as if the Positive control did not amplify, therefore the Problem has occurred during the setup of the PCR assay reaction.

3. How should it be interpreted if only the PCR control (PCRC) showed amplification but neither the *S. uberis* target nor the Isolation Control (IsoC) amplified for a sample?

- This indicates a poor isolation. The isolation procedure must be repeated.

4. How should it be interpreted if only the Isolation Control (IsoC) was amplified in a sample?

- The sample tested can be considered as *S. uberis* negative.

5. How should it be interpreted if only the *S. uberis* target and the PCR control (PCRC) were amplified in a sample?

- The sample tested can be considered as *S. uberis* positive.

6. How should it be interpreted if only the *S. uberis* target was amplified in a sample?

- The sample tested should be considered as *S. uberis* positive. At high *S. uberis* cell input, the *S. uberis* amplicon will be predominant and thus the PCR control (PCRC) as well as the Isolation Control (IsoC) may not amplify as they compete for PCR resources.

7. How should it be interpreted if only the PCR control (PCRC) and the Isolation Control (IsoC) showed amplification in a sample?

- The sample tested can be considered negative

8. Can I freeze and thaw the provided enzymes for DNA isolation?

- Repeated freeze/thaw of the reconstituted Proteinase K and Lysozyme will reduce the activity of the enzymes and hence the isolation efficiency. The result is lower DNA yield. It is recommended to divide the reconstituted enzymes into smaller working aliquots prior to freezing.

9. What If my incubation temperature during extraction varied from the specified 37°C or 55°C for Lysozyme and Proteinase K, respectively?

- At other temperatures the activity of both the Proteinase K and Lysozyme will be reduced. This will result in a reduction in your DNA yields.

10. What If my incubation time varied from the 45 minutes specified in the product manual?

- Less than 45 minutes will result in a lower DNA yields. More than 45 minutes may not affect your DNA yields.

11. What If I forgot to do a dry spin after my second wash?

- Your first DNA elution will be contaminated with the Wash Solution. This may dilute the DNA yield in your first elution and it may interfere with the PCR detection, as ethanol is known to be a PCR inhibitor.

12. What If I forgot to add Isolation Control (IsoC) during the Isolation?

- It is recommended that the isolation is repeated.

Reference:

B. E. Gillespie and S. P. Oliver. 2005. Simultaneous Detection of Mastitis Pathogens, *Staphylococcus aureus, Streptococcus uberis*, and *Streptococcus agalactiae* by Multiplex Real-Time Polymerase Chain Reaction. **J. Dairy Sci.** 88:3510–3518.

Related Products	Product #
Milk Bacterial DNA Isolation Kit	21500
Bacterial Genomic DNA Isolation Kit	17900

Technical Assistance

NORGEN's Technical Service Department is staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of NORGEN products. If you have any questions or experience any difficulties regarding Norgen's Urine DNA Isolation Mini Kit (Slurry Format) or NORGEN products in general, please do not hesitate to contact us.

NORGEN customers are a valuable source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at NORGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362. or call one of the NORGEN local distributors (www.norgenbiotek.com) or through email at techsupport@norgenbiotek.com.

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